2

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/87, A61K 47/48, C12N 15/11, 9/00, A61K 31/70

A1

(11) International Publication Number: WO 94/23050

(43) International Publication Date: 13 October 1994 (13.10.94)

(21) International Application Number: PCT/US94/03643

(22) International Filing Date: 4 April 1994 (04.04.94)

(30) Priority Data:
08/042,943 5 April 1993 (05.04.93) US

(71) Applicants: UNIVERSITY OF CONNECTICUT [US/US]; Storrs, CT 06269 (US). TARGETECH, INC. [US/US]; 290 Pratt Street, Meriden, CT 06450 (US).

(72) Inventors: CHIOU, Henry, C.; 87 Maplewood Road, Southington, CT 06489 (US). INNAIMO, Steven, F.; 134 Plainfield Drive, Oakville, CT 06779 (US). WU, George, Y.; 52 Ridgebury Road, Avon, CT 06001 (US).

(74) Agents: DECONTI, Giulio, A. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: TARGETED DELIVERY OF GENES ENCODING ANTISENSE POLYRIBONUCLEOTIDES

(57) Abstract

Molecular complexes for targeting a gene encoding an antisense polyribonucleotide to a specific cell *in vivo* obtaining production of the polyribonucleotide within the targeted cell, and effecting specific inhibition of the expression of cellular or noncellular genes are disclosed. An expressible gene encoding a desired antisense polyribonucleotide is complexed to a conjugate of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent is specific for a cellular surface structure which mediates internalization of ligands by endocytosis. An example is the asialoglycoprotein receptor of hepatocytes. The gene-binding agent is a compound such as a polycation which stably complexes the gene under extracellular conditions and releases the gene under intracellular conditions so that it can function within a cell. The molecular complex is stable and soluble in physiological fluids and can be used in antisense gene therapy to selectively transfect cells *in vivo* or *in vitro* to provide for production of the antisense polyribonucleotide within the targeted cell, and inhibition of the expression of cellular or noncellular genes.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

TARGETED DELIVERY OF GENES ENCODING ANTISENSE POLYRIBONUCLEOTIDES

Background of the Invention

Antisense polynucleotides are a means of specifically inhibiting unwanted gene expression in cells. They can be used to hybridize to and inhibit the function of an RNA, typically a messenger RNA, by physically blocking the binding of ribosomes or other proteins, thus preventing translation of the mRNA. Antisense polynucleotides also include RNAs with catalytic activity (ribozymes), which can selectively bind to complementary sequences on a target RNA and physically destroy the target by mediating a cleavage reaction.

Antisense polynucleotides can be in the form of small, chemically synthesized DNA or RNA oligonucleo- tides, or can be larger RNAs, such as mRNAs, biosynthetically generated in vitro or in vivo by transcription of an antisense gene. Since hundreds of copies of RNA can be synthesized from each copy of a gene, a few molecules of an antisense gene in a cell would achieve the same effect as the introduction into the cell of a large number of antisense oligonucleo- tides. The 50 to 300 times greater size of a typical mRNA allows an antisense mRNA to bind with greater affinity and specificity compared to an oligonucleo- tide. In addition, unlike oligonucleotides, a plasmid-borne gene can incorporate a wide variety of supplemental DNA sequences to enhance or modulate the expression of the antisense polyribonucleotide. For example, inclusion of origin sequences which direct episomal replication of a plasmid and promoter and/or enhancer sequences that sustain a high level of expression over long periods would allow long term production of an antisense polyribonucleotide. This strategy would be particularly suited for inhibiting the constitutive expression of a cellular gene or for treating chronic conditions or diseases. To achieve a similar effect with an antisense oligonucleotide would most likely require continuous or repeated intravenous administration or highly stable antisense constructs.

Summary of the Invention

This invention pertains to a soluble molecular complex for targeting a gene encoding an RNA transcript to a specific cell in vivo or in vitro and obtaining production of the RNA within the targeted cell. The molecular complex comprises an expressible gene encoding a desired polyribonucleotide complexed to a carrier which is a conjugate of a cell-specific binding agent and a gene-binding agent. In antisense applications, the RNA transcribed from the delivered gene can be used to hybridize to and inhibit the function of an RNA contained within the cell. The target RNA is typically a messenger RNA. The RNA transcribed from the delivered gene can also be an RNA with catalytic activity (a ribozyme), which can selectively destroy the target RNA. The target for antisense or ribozyme-mediated inhibition

can be a gene or genes of cellular origin (e.g., a cellular oncogene) or of noncellular origin (e.g., a viral oncogene or the genes of an infecting pathogen such as a virus or a parasite such as malaria, trypanosome, lysteria, or mycoplasma).

The cell-specific binding agent is specific for a cellular surface structure, typically a receptor, which mediates internalization of bound ligands by endocytosis, such as the asialoglycoprotein receptor of hepatocytes. The cell-specific binding agent can be a natural or synthetic ligand (for example, a protein, polypeptide, glycoprotein, etc.) or it can be an antibody, or an analogue thereof, which specifically binds a cellular surface structure which then mediates internalization of the bound complex. The gene-binding component of the conjugate is a compound such as a polycation which stably complexes the gene under extracellular conditions and releases the gene under intracellular conditions so that it can function within the cell.

The complex of the gene and the carrier is stable and the carrier is stable and soluble in physiological fluids. It can be administered in vivo where it is selectively taken up by the target cell via the surface-structure-mediated endocytotic pathway. The incorporated gene expressed and the gene-encoded product accumulates within the transfected cell.

The soluble molecular complex of this invention can be used to specifically transfect cells in vivo or in vitro to provide for synthesis of a desired product. This selective transfection is useful for antisense gene therapy and other applications which require selective genetic alteration of cells to inhibit the expression of cellular or foreign genes. The RNA transcript produced from the delivered gene hybridizes with its complementary RNA, inhibiting its function either by steric hindrance, or by physical cleavage, thereby blocking expression of the target gene or genes.

Brief Description of the Drawings

Figure 1 is a schematic depiction of the construction of a plasmid encoding an antisense RNA directed against hepatitis B surface antigen (pJ3Ω0.8HTD1) and a plasmid encoding an antisense RNA directed against the hepatitis core antigen gene as well as the DR1 and polyadenylation site.

Figure 2 shows the reduction of HBsAg mediated by 21-mer antisense oligodeoxynucleotide and antisense mRNA-generating plasmid pJ3Ω0.8HTD1 (Anti-C).

Detailed Description of the Invention

A soluble, targetable molecular complex is used to selectively deliver a gene encoding a polyribonucleotide to a target cell or tissue in vivo or in vitro. The molecular complex comprises the gene to be delivered complexed to a carrier made up of a binding agent specific for the target cell and a gene-binding agent specific for the target cell and a gene-binding agent. The complex is selectively taken up by the target cell and the polyribonucleotide is produced therein.

The gene, generally in the form of DNA, encodes the desired polyribonucleotide. Typically, the gene comprises a sequence encoding the polyribonucleotide in a form suitable for transcription and post-transcriptional processing by the target cell. For example, the gene is linked to appropriate genetic regulatory elements required for transcription of the gene by a cellular RNA polymerase and processing of the primary RNA transcript by cellular proteins into a stable form of RNA, such as mRNA. These include promoter and enhancer elements operable in the target cell, as well as other elements such as polyadenylation signals and splicing signals, which determine the internal and 3'-end structure of the RNA. The gene can be contained in an expression vector such as a plasmid or a transposable genetic element along with the genetic regulatory elements necessary for transcription of the gene.

The carrier component of the complex is a conjugate of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent specifically binds a cellular surface structure which mediates its internalization by, for example, the process of endocytosis. The surface structure can be a protein, polypeptide, carbohydrate, lipid or combination thereof. It is typically a surface receptor which mediates endocytosis of a ligand. Thus, the binding agent can be a natural or synthetic ligand which binds the receptor. The ligand can be a protein, polypeptide, glycoprotein or glycopeptide which has functional groups that are exposed sufficiently to be recognized by the cell surface structure. It can also be a component of a biological organism such as a virus, cells (e.g., mammalian, bacterial, protozoan) or artificial carriers such as liposomes.

The binding agent can also be an antibody, or an analogue of an antibody such as a single chain antibody, which binds the cell surface structure.

Ligands useful in forming the carrier will vary according to the particular cell to be targeted. For targeting hepatocytes, glycoproteins having exposed terminal carbohydrate groups such as asialoglycoprotein (galactose-terminal) can be used, although other ligands such as polypeptide hormones may also be employed. Examples of asialoglycoproteins include asialoorosomucoid, asialofetuin and desialylated vesicular stomatitis virus. Such ligands can be formed by chemical or enzymatic desialylation of glycoproteins that possess terminal sialic acid and penultimate galactose residues. Alternatively, asialoglycoprotein ligands can be formed by coupling galactose terminal carbohydrates such as lactose or arabinogalactan to non-galactose bearing proteins by reductive lactosamination.

For targeting the molecular complex to other cell surface receptors, other types of ligands can be used, such as mannose for macrophages (lymphoma), mannose-6-phosphate glycoproteins for fibroblasts (fibrosarcoma), intrinsic factor-vitamin B12 or bile acids for enterocytes and insulin for fat cells. Alternatively, the cell-specific binding agent can be a receptor or receptor-like molecule, such as an antibody which binds a ligand (e.g., antigen) on the cell surface. Such antibodies can be produced by standard procedures.

The gene-binding agent complexes the gene to be delivered. Complexation with the gene must be sufficiently stable <u>in vivo</u> to prevent significant uncoupling of the gene

extracellularly prior to internalization by the target cell. However, the complex is cleavable under appropriate conditions within the cell so that the gene is released in functional form. For example, the complex can be labile in the acidic and enzyme rich environment of lysosomes. A noncovalent bond based on electrostatic attraction between the gene-binding agent and the expressible gene provides extracellular stability and is releasable under intracellular conditions.

Preferred gene-binding agents are polycations that bind negatively charged polynucleotides. These positively charged materials can bind noncovalently with the gene to form a soluble, targetable molecular complex which is stable extracellularly but releasable intracellularly. Suitable polycations are polylysine, polyarginine, polyornithine, basic proteins such as histones, avidin, protamines and the like. A preferred polycation is polylysine (e.g., ranging from 3,800 to 60,000 daltons). Other noncovalent bonds that can be used to releasably link the expressible gene include hydrogen bonding, hydrophobic bonding, electrostatic bonding alone or in combination such as, anti-polynucleotide antibodies bound to polynucleotide, and strepavidin or avidin binding to polynucleotide containing biotinylated nucleotides.

The carrier can be formed by chemically linking the cell-specific binding agent and the gene-binding agent. The linkage is typically covalent. A preferred linkage is a peptide bond. This can be formed with a water soluble carbodiimide as described by Jung, G. et al. (1981) Biochem. Bioshys. Res. Commun. 101::599-606. An alternative linkage is a disulfide bond.

The linkage reaction can be optimized for the particular cell-specific binding agent and gene-binding agent used to form the carrier. Reaction conditions can be designed to maximize linkage formation but to minimize the formation of aggregates of the carrier components. The optimal ratio of cell-specific binding agent to gene-binding agent can be determined empirically. When polycations are used, the molar ratio of the components will vary with the size of the polycation and the size of the gene- binding agent. In general, this ratio ranges from about 10:1 to 1:1, preferably about 5:1. Uncoupled components and aggregates can be separated from the carrier by molecular sieve or ion exchange chromatography (e.g., Aquapore™ cation exchange, Rainin).

In one embodiment, asialoorosomucoid-polylysine conjugate is formed with the crosslinking agent 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide. After dialysis, the conjugate is separated from unconjugated components by preparative acid-urea polyacrylamide gel electrophoresis (pH 4-5). The conjugate can be further purified on the carboxymethyl functionalized column (Waters AP-1 column). See U.S. Patent Application Serial No. 08/043,008, filed on April 5, 1992, the teachings of which are incorporated by reference herein.

The gene encoding the antisense construct can be complexed to the carrier by a stepwise dialysis procedure. In a preferred method, for use with carriers made of polycations

such as polylysine, the dialysis procedure begins with a 2M NaCl dialyzate and ends with a .15M NaCl solution. The gradually decreasing NaCl concentration results in binding of the gene to the carrier. In some instances, particularly when concentrations of the gene and carrier are low, dialysis may not be necessary; the gene and carrier are simply mixed and incubated.

The molecular complex can contain more than one copy of the same gene or one or more different genes. Preferably, the weight ratio of gene to the carrier is from about 1:5 to 5:1, preferably about 1:2 (approximate molar ratio 1:100 to 1:200). The appropriate ratio for a particular polynucleotide and carrier can be determined by the gel retardation assay described in U.S. Patent No. 5,166,320.

The molecular complex of this invention can be administered parenterally. Preferably, it is injected intravenously. The complex is administered in solution in a physiologically acceptable vehicle.

Cells can be transfected in vivo for transient production of the polyribonucleotide. For prolonged production, the gene can be administered repeatedly. Alternatively, the transfected target cell can be stimulated to replicate by surgical or pharmacological means to prolong the activity of the incorporated gene. See, for example, U.S. Patent Application Serial No. 588,013, filed September 25, 1990, the teachings of which are incorporated by reference herein. Drugs that disrupt translocation or fusion of endosomes to lysosomes such as colchicine or taxol can be used to prolong expression. See U.S. Patent Application Serial No. 950,789, filed September 24, 1992, the teachings of which are incorporated by reference herein.

Delivery of the gene can be enhanced by coupling the carrier to a virus such as adenovirus. See U.S. Patent Applicaton Serial No. 950,453, filed September 24, 1992 the teachings of which are incorporated by reference herein.

The method of this invention can be used to selectively deliver a gene to a target cell in vivo for antisense gene therapy or other applications which require inhibition of the expression of specific cellular or foreign genes. The RNA transcript produced from the delivered gene hybridizes with its complementary RNA, inhibiting its function either by steric hindrance, or by physical cleavage, thereby blocking expression of the target gene or genes. For example, the gene can be targeted to specific cells to alleviate a genetic abnormality caused by overexpression of a cellular or viral oncogene. In addition, the method can be used to treat negative dominant genetic diseases in which an abnormal gene product interferes with a normal protein. For example, the method can be used to deliver an antisense or ribozyme directed against the abnormal fibrillin produced in Marfan's syndrome or against the abnormal collagen produced in osteogenesis imperfecta type I. The method can also be used to inhibit the expression of the genes of an infecting pathogen such as a virus (hepatitis, HIV) or a parasite such as malaria, trypanosome, lysteria, or mycoplasma. For example, hepatitis genes such as the genes encoding one or more of the surface or core

antigens can be assembled in an expression vector in reverse orientation to generate an antisense transcript which blocks translation of the corresponding genes.

The molecular complex of this invention is adaptable for delivery of a wide range of genes to a specific cell or tissue. In a preferred embodiment, the complex is targeted to the liver by exploiting the hepatic asialoglycoprotein receptor system which allows for in vivo transfection of hepatocytes by the process of receptor-mediated endocytosis.

The method of the invention can be used to treat virus infections of liver cells. This includes infections by any of the liver-specific hepatitis viruses. Infection by human hepatitis B virus often results in a chronic, persistent infection. This form of viral infection is more suitably treated by antisense gene therapy compared to antisense oligonucleotide therapy. Because the hepatitis B virus genome is very small, coding for only three extensively overlapping RNA transcripts, antisense genes can be engineered to encode an RNA that can hybridize to one or more large regions common to all of the viral transcripts. In a preferred embodiment, a complex can be used to deliver a plasmid-borne antisense gene specifically to chronically infected hepatocytes to block the production of hepatitis B virus. The gene can code for the production of an antisense RNA transcript that hybridizes to all of the RNAs produced by the hepatitis B virus, inhibiting production of all viral polypeptides. The resulting soluble complex is administered parenterally to target liver cells of the individual afflicted with the virus in amounts sufficient to selectively transfect the cells and to provide sufficient production of the antisense RNA to achieve inhibition of virus production.

The invention is illustrated further by the following exemplification.

EXEMPLIFICATION

Materials and Methods

Plasmid DNA Construction

The 9.4 Kb plasmid pAdw-HTD, which contains two head-to-tail copies of the hepatitis B virus (HBV) genome, was provided by T.J. Liang (Massachusetts General Hospital, Boston, MA). The first construct termed pJ3Ω1.0HTD1 or "Anti S" was created by first digesting pAdw-HTD with the restriction endonucleases EcoRI and EcoRV followed by subsequent isolation of 1044bp fragment by agarose gel electrophoresis and glass bead extraction (Geneclean II®, Bio101, LaJolla, CA). This fragment spans most of the pre-S2 signal peptide gene and the complete surface antigen gene but not the surface antigen promoter region. This EcoRI/EcoRV fragment was then ligated into the EcoRI and SmaI sites within the polylinker region of the expression vector PJ3Ω (ATCC, 37719, Nuc. Acids Res. 18:1068, 1990) in such a fasion that transcription, driven by the SV40 early promoter, would produce a 1Kb antisense strand of messenger RNA (mRNA) that could then bind to its

complimentary sequence in the HBV pregenomic mRNA and inihibit translation of HBV surface antigen, thereby disrupting viral assembly (see Figure 1).

The second construct, termed pJ3Ω0.8HTD3 or "Anti C" was designed to generate a 0.8 Kb antisense mRNA complementary to the region in the HBV pregenomic mRNA which encodes the precore and core antigens. Core antigen, like the surface antigen, is a coat protein that is essential for viral packaging. Also included in he Anti C fragment is the 11bp direct repeat sequence, denoted DR1, this is critically involved in the initiation of viral DNA synthesis, as well as the unique cleavage/polyadenylation signal specifying the common 3' termini of all HBV RNA species (Ganem and Varmus (1987) Ann. Rev. Biochem. 56:651-693). As a result, antisense mRNA transcribed from this region should block all viral protein synthesis as well as replication. To construct pJ3Ω0.8HTD3, pAdw-HTD was cleaved with the restriction endonucleases Fspl and Apal to generate a 802bp fragment which was separated by agarose gel electrophoresis and isolated using Geneclean II®. The Anti C fragment was ligated into the Apal/Smal sites within the polylinker region of the cloning vector pGEM-7zf(+) (Promega, Madison, WI). The Anti C fragment was then subcloned into the Clal and Smal sites within the polylinker region of the pJ3Ω vector. To accomplish this, the pGEM clone was first cut with Apal and blunted by the addition of the large Klenow fragment of DNA polymerase I plus 200mM dNTP mixture. Blunting of the Apal end was followed by digestion with Clal to yield a 818bp fragment which was then ligated into the pJ3Ω expression vector (Figure 1).

Competent DH5α E. coli (Gibco BRL) were transformed with the plasmid constructs according to standard protocols (Sambrook et al. (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, 2nd ed.). Large scale preparations of the plasmid DNA were carried out by standard procedures.

Cells and Cell Culture

Human hepatoma, HepG2 .2.15 cells (provided by Dr. George Acs, Albert Einstein College of Medicine, Bronx, NY) were maintained and grown in minimum essential medium (MEM) supplemented with 0.1mM non-essential amino acids, 0.1mM sodiumpyruvate, 2mM L-glutamine, 50 units/ml penicillin, 50μg/ml streptomycin and 10% fetal calf serum. HepG2 .2.15 are clonal cells derived from HepG2 cells transformed with a plasmid containing the HBV genome. The constitutively produce and secrete hepatitis B surface antigen particles, nucleocapsids and virions (Acs et al. (1987) Proc. Natl. Acad. Sci. USA 84:4641-4644).

Preparation of Targetable Antisense mRNA-Generating Plasmid and Oligonucleotide DNA

Antisense plasmid pJ3Ω0.8HTD3 and a synthetic 21-mer antisense oligodeoxynucleotide (Synthetic Genetics, San Diego, CA) complimentary to the region in the human hepatitis B virus (ayw subtype) genome that encodes the polyadenylation signal, were titrated with asialoorosomucoid (ASOR)-polylysine conjugates to form soluble

complexes using an agarose gel retardation assay as described by Wu and Wu (1987) J. Biol. Chem. 262:4429-4432; U.S. Patent No. 5,166,320. For these experiments, a ratio of 0.8:1 by weight (ASOR-polylysine conjugate:DNA), which allowed full retardation of the DNA, was selected and used to prepare the antisense plasmid DNA complex while a ration of 1.6:1 by weight was selected for preparation of the antisense oligonucleotide complex.

Assay of Anitisense Plasmid and Oligonucleotide Activity Delivered to Cells via Receptor-Mediated Endocytosis

To determine the effect on viral gene expression of the antisense mRNA transcribed by the Anti C construct, HepG2 .2.15 cells were seeded at $3.5x10^6$/60mm dish 24 hours prior to use. Preceding transfection, all cells were treated with 100μM chloroquine for one hour, followed by 3 washes with phosphate-buffered saline. Cells were then overlaid with fresh MEM containing 100μg of the antisense plasmid in complex or 690μg of antisense oligonucleotide in complex plus enough calcium chloride to increase the Ca^{++} concentration 2mM followed by incubation at 37°C and 5% CO_2 (a slight modification of the protocol of Wu and Wu (1988) Biochemistry 27:887-892). As controls, some cells were left untransfected, transfected with 100μg of antisense plasmid DNA alone, or with 690μg of antisense oligonucleotide alone. To determine baseline hepatitis B virus surface antigen (HBsAg) levels, an ELISA (Abbott) assay was performed on 50μl samples of medium removed from each plate prior to transfection and assayed according to the procedure described by the manufacturer. 50μl samples of medium were subsequently removed from each dish every day for 6 days and processed for HBsAg.

RESULTS

pJ3Ω0.8HTD3 was introduced into HepG 2.2.15 cells via asialoglycoprotein receptor mediated endocytosis in order to examine the plasmid's ability to inhibit production of HBsAg. As a reference for comparison some cells were also treated with an antisense 21-mer oligodeoxynucleotide directed against the unique HBV polyadenylation signal sequence. This oligodeoxynucleotide was idential to the one used by Wu and Wu (1992) J. Biol. Chem. 267:12436-12439 to inhibit HepG 2.2.15 HBsAg expression. A significant reduction in HBsAg expression, relative to untreated cells, was observed in cells that received either pJ3Ω0.8HTD3-polylysine-ASOR complex or the antisense oligo-polylysine-ASOR complex (Figure 2). In both cases the inhibition persisted for a least 6 days. Reduction of HBsAg was not seen in cells treated with plasmid or oligodeoxynucleotide alone, suggesting that the ASOR-polylysine conjugate was necessary to deliver these DNAs to their intracellular sites of action. In a separate experiment, cells treated with a pJ3Ω1.0HTD1-complex exhibited a similar reduction in HBsAg levels compared to cells treated with pJ3Ω0.8HTD3-complex (data not shown).

As figure 2 shows pJ3Ω0.8HTD3 was as effective as the antisense oligonucleotide at inhibiting surface antigen expression, both in terms of the level of inhibition as well as its duration. However, the total amount of plasmid added to the cells was almost 7-fold less than that of the anti-HBV oligonucleotide. Calculated on a molar basis, each molecule of pJ3Ω0.8HTD3 was roughly equivalent to 1400 molecules of the antisense oligonucleotide in inhibiting HBsAg expression. We expect that this was due to the ability of the plasmid to generate many copies of its antisense transcript once it was delivered into the cell. It is also possible that differences in stability, site of action, intracellular retention, or differences in the properties of the complexes made with each type of DNA may have contributed to this effect. This illustrates a potential advantage of plasmid based antisense systems compared to oligonucleotides. A smaller amount of DNA needs to be delivered to the target cel in order to achieve a therapeutic dose. In addition, a plasmid contains cis-acting sequences, such as promoters, enhances, polyadenylation sites, origins of replication, etc. which directed or influence the expression of its RNA. These sequences can be modified or substituted in order to tailer expression for specific circumstances. For example, one could incorporate an inducible promoter into the plasmid in order to activate expression of the antisense mRNA only at certain times or under certain conditions. Or, one could achieve a very short term, transient inhibition by use of consensus RNA destabilizing elements within the 3' untranslated region of the mRNA. Another possibility would be to produce a sustained antisense mediated inhibition by incorporated sequences into the plasmid which would allow it to be maintained episomally within the cell. Many other characteristics could also be incorporated into a plasmid-based antisense system, thus allowing for a great deal of flexibiltiy and control in its use.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

CLAIMS

1. A soluble molecular complex for targeting a gene encoding an antisense RNA to a specific cell, the complex comprising an expressible gene encoding an RNA which hybridizes to and inhibits the function of a cellular RNA, the RNA being complexed with a carrier comprising a cell-specific binding agent and a gene-binding agent.

2. A soluble complex of claim 1, wherein the antisense RNA is directed to the RNA transcript of a viral or cellular oncogene.

3. A soluble complex of claim 1, wherein the antisense RNA is directed to an RNA transcript of a pathogen.

4. A soluble complex of claim 1, wherein the pathogen is a virus.

5. A soluble complex of claim 1, wherein the virus is hepatitis.

6. A soluble complex of claim 1, wherein the virus is HIV.

7. A soluble complex of claim 1, wherein the antisense is directed against a gene associated with a dominant negative genetic disorder.

8. A soluble complex of claim 1, wherein the antisense RNA is a ribozyme.

9. A soluble molecular complex of claim 1, wherein the gene-binding agent is a polycation.

10. A soluble molecular complex of claim 8, wherein the polycation is polylysine.

11. A soluble molecular complex of claim 1, wherein the cell-specific binding agent binds a surface receptor of the cell which mediates endocytosis.

12. A therapeutic composition comprising a solution of the molecular complex of claim 1 in a physiologically acceptable vehicle.

13. A soluble molecular complex for targeting a gene encoding an antisense RNA to a hepatocyte, the complex comprising an expressible gene encoding the antisense RNA

specifically hybridizable to an RNA transcript produced in the hepatocyte complexed with a carrier of a ligand for the asialoglycoprotein receptor and a polycation.

14. A soluble molecular complex of claim 13, wherein the antisense RNA is directed against an RNA transcript of hepatitis virus.

15. A soluble molecular complex of claim 13, wherein the polycation is polylysine.

16. A soluble molecular complex of claim 13, wherein the gene is contained in an expression vector along with genetic regulatory elements necessary for expression of the gene by the hepatocyte.

17. A soluble molecular complex of claim 16, wherein the expression vector is a plasmid or viral DNA.

18. A soluble molecular complex of claim 13, wherein the antisense RNA is a ribozyme.

19. A therapeutic composition comprising a solution of the molecular complex of claim 13, in a physiological vehicle.

20. A method of blocking translation of an RNA transcript in a cell of an organism, comprising administering to an organism a soluble molecular complex comprising an expressible gene encoding an RNA which hybridizes to and inhibits trhe function of a cellular RNA, the RNA being complexed with a carrier comprising a cell-specific binding agent and a gene-binding agent.

21. A method of claim 20, wherein the antisense RNA is directed to the RNA transcript of a viral or cellular oncogene.

22. A method of claim 20, wherein the antisense RNA is directed to a transcript of a pathogen.

23. A method of claim 20, wherein the pathogen is hepatitis virus.

24. A method of claim 20, wherein the gene-binding agent is a polycation.

25. A method of claim 24, wherein the polycation is polylysine.

26. A method of claim 20, wherein the cell-specific binding agent binds a surface receptor of the cell which mediates endocytosis.

27. A method of claim 20, wherein the ligand is an asialoglycoprotein and the targeted cell is a hepatocyte.

28. A method of claim 20, wherein the antisense RNA is a ribozyme.

29. A method of claim 20, wherein the molecular complex is administered intravenously.

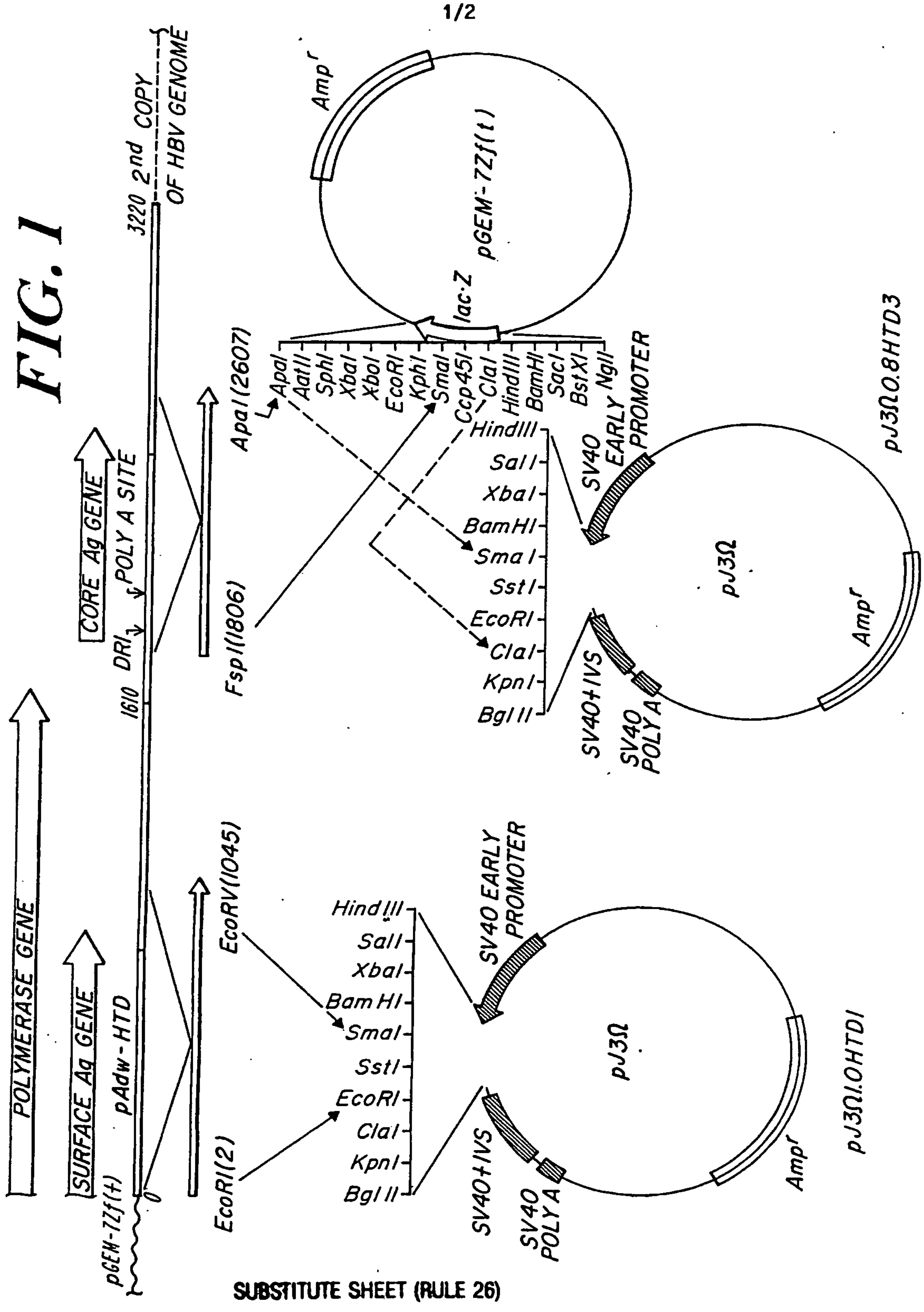
POLYMERASE GENE
SURFACE Ag GENE
CORE Ag GENE
pAdw-HTD
pGEM-7Zf(+)
0
1610
3220
DRI
POLY A SITE
2nd COPY OF HBV GENOME
EcoRI(2)
EcoRV(1045)
FspI(1806)
ApaI(2607)
Ampr
pGEM-7Zf(+)
lac Z
ApaI
AatII
SphI
XbaI
XhoI
EcoRI
KpnI
SmaI
Ccp45I
ClaI
HindIII
BamHI
SacI
BstXI
NglI
HindIII
SalI
XbaI
BamHI
SmaI
SstI
EcoRI
ClaI
KpnI
BglII
SV40 EARLY PROMOTER
SV40+IVS
SV40 POLY A
pJ3Ω
Ampr
pJ3Ω0.8HTD3
pJ3Ω1.0HTD1

FIG. 1

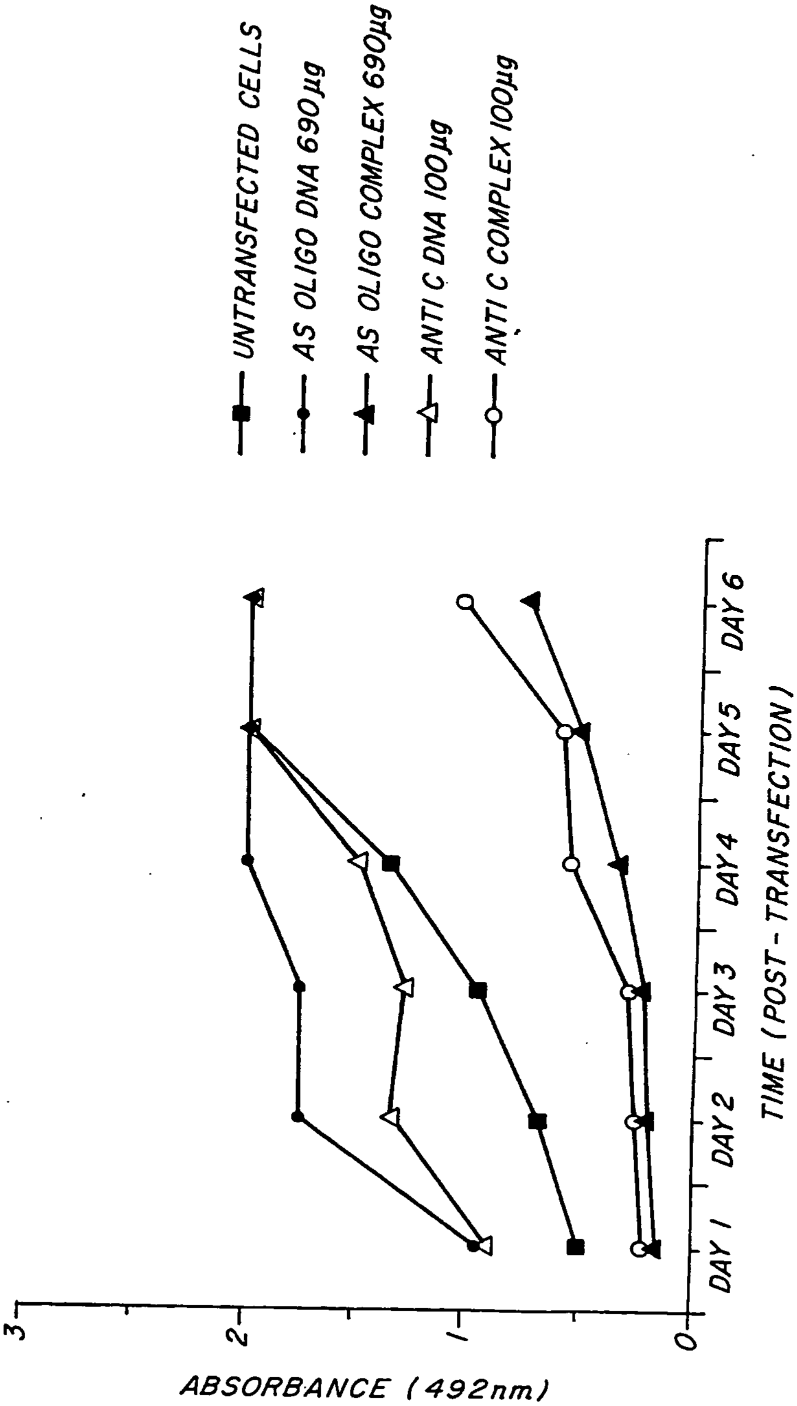
UNTRANSFECTED CELLS
AS OLIGO DNA 690µg
AS OLIGO COMPLEX 690µg
ANTI C DNA 100µg
ANTI C COMPLEX 100µg
ABSORBANCE (492nm)
0
1
2
3
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
TIME (POST-TRANSFECTION)

FIG. 2

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/US 94/03643

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/87 A61K47/48 C12N15/11 C12N9/00 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 04701 (UNIVERSITY OF CONNECTICUT) 18 March 1993 see page 2, line 1 - page 25, line 8 see claims ---	1-29
X	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 267, no. 18 , 25 June 1992 , BALTIMORE, MD US pages 12436 - 12439 WU, G. & WU, C. 'Specific inhibition of hepatitis B viral gene expression in vitro by targeted antisense oligonucleotides' cited in the application see the whole document --- -/--	1,3-5, 9-17,19, 20, 22-27,29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 July 1994	22. 07. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Andres, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 05250 (UNIVERSITY OF CONNECTICUT) 2 April 1992 cited in the application see page 2, line 14 - line 28 see page 4, line 1 - page 7, line 7 see examples see claims ---	1-29
Y	JOURNAL OF THE NATIONAL CANCER INSTITUTE vol. 81, no. 20 , 18 October 1989 , BETHESDA, MD, USA pages 39 - 44 ROTHENBERG, M. ET AL. 'Oligodeoxynucleotides as anti-sense inhibitors of gene expression: therapeutic implications' see the whole document ---	1-7, 9-17, 19-27,29
Y	WO,A,92 06693 (FOX CHASE CANCER CENTER) 30 April 1992 see claims ---	8,18,28
A	J BIOL CHEM 264 (29). 1989. 16985-16987 WU, C. ET AL. 'TARGETING GENES DELIVERY AND PERSISTENT EXPRESSION OF A FOREIGN GENE DRIVEN BY MAMMALIAN REGULATORY ELEMENTS IN-VIVO.' see the whole document ---	1-29
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 87 , May 1990 , WASHINGTON US pages 3410 - 3414 WAGNER, E. ET AL. 'Transferrin-polycation conjugates as carriers for DNA uptake into cells' see the whole document -----	1-29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/03643

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 20-29 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. ...al Application No
PCT/US 94/03643

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9304701	18-03-93	AU-A-	2678092	05-04-93
WO-A-9205250	02-04-92	AU-A-	8628291	15-04-92
		CA-A-	2092319	26-03-92
		EP-A-	0556197	25-08-93
		JP-T-	6503714	28-04-94
WO-A-9206693	30-04-92	AU-A-	8957591	20-05-92
		CA-A-	2094608	23-04-92
		EP-A-	0554376	11-08-93
		JP-T-	6502311	17-03-94